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13. ABSTRACT (Maximum 200 words) We previously showed that micronucleation provides an effective means of removing extrachromosomally amplified genes from tumor cells, and for reducing tumorigenicity through induction of differentiation or apoptosis. The research conducted in the past year was designed to determine the mechanisms of micronucleation and to identify new agents that could induce this process. We report that micronuclei can be generated during S-phase by nuclear budding. This represents a process that has not been reported previously. We show that micronucleation can be induced by a broad spectrum of antiproliferative agents that impede replication fork progression. Micronucleation occurred at low frequency in cells with a functional p53 pathway, but eliminating p53 function substantially increased micronucleation efficiency. Since p53 deficiency and gene amplification occur in vivo only in tumors, the results indicate that treatments that induce micronuclei should be highly selective for neoplasms. We also show that micronuclei are generated during tumor growth in vivo, and preliminary results indicate that purification of such structures directly from disaggregated tumor tissue may provide a diagnostic method for DMs and for specifying the chromosomal location(s) from which they arose.				
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Introduction

Loss of genetic stability occurs frequently during the development of human neoplasms. One consequence of genetic instability is loss of gene copy number control, which can be manifested by increases or decreases in entire chromosomes, or parts of chromosomes. In the latter case, local increases in gene copy number, gene amplification, is manifested in as many as 50% of human tumors by the presence of autonomously replicating, acentric, extrachromosomal chromatin bodies called double minute chromosomes (DMs) (1). Such structures have never been observed in normal cells due to the existence of control mechanisms that effectively limit their formation (2, 3). DMs encode proteins involved in various facets of signal transduction and cell cycle control, and the overexpression of such gene products can enhance cell growth or survival (4, 5, 6, 7). We have sought to develop techniques to isolate DMs to ascertain the putative oncogenes they encode, and to eliminate them from tumor cells as a potential means of selectively arresting tumor cell growth (8).

We have shown that DMs can be removed from the cell nucleus by a process involving formation of subnuclear bodies called micronuclei (4, 8, 9). Micronuclei selectively capture acentric fragments such as DMs, and can result in a sufficient reduction in DM copy number to reduce tumorigenicity in some cell lines (4), or induce differentiation or apoptosis in others (6, 7). We also were able to develop a rapid and highly effective procedure to purify micronuclei and the DMs they contain (8). The acentric DNA was of sufficient purity to enable production of probes for fluorescence in situ hybridization (FISH) from cells containing an average of only ~2-4DMs per cell (8).

These data provided the incentive to elucidate the mechanisms of micronucleation to aid in identifying or screening for drugs that might be more effective at eliminating DMs from cancer cells in vivo. Four goals were included in a modified statement of work for 1996. First, agents other than hydroxyurea were to be analyzed for micronucleation capacity, and their cell cycle consequences were to be studied to gain insight into potential micronucleation mechanisms. Second, we proposed to extend the micronucleation purification procedure developed for cell lines in vitro to tumors growing in vivo. Third, we proposed to build recombinant vectors to enable us to investigate the hypothesis that tumor evolution might be expedited by the transfer of genetic information mediated by micronuclei. A final goal, if time and resources permitted, was to assess the phenotypic changes induced in different cell lines upon removal of extrachromosomally amplified genes.

Methods and Results

I. Pharmacologic specificity of micronucleating agents and potential mechanisms

A. Conditions that induce micronucleation

We found previously that micronuclei are induced in many cell lines in response to concentrations of hydroxyurea (HU) that slow but do not completely inhibit DNA replication (4, 10). We expanded this analysis by studying the effects of many inhibitors of DNA replication on micronucleation efficiency in a colon cancer cell line of neuroendocrine origin (COLO 320DM). Cells were treated with different concentrations of the inhibitors cited in Figure 1. The number of micronuclei generated and the consequences of each inhibitor on cell cycle progression were determined. Cell cycle analyses were performed by labeling cells with the DNA stain propidium iodide to determine DNA content per cell, and cells in S-phase were determined directly by pulse labeling with the DNA precursor bromodeoxyuridine (BrdU; see (11) for detailed methods). The data summarized in Figure 1 show that micronucleation efficiency was increased by all agents when used at concentrations that elongate S-phase. Interestingly, the micronucleation efficiency dropped when the agents were used at higher concentrations. These studies significantly expand the pharmacologic agents potentially suitable for micronucleation in vivo.

B. Micronucleation occurs during S-phase

The inhibitor studies summarized above are consistent with two models of how micronuclei could be generated (see Figure 2). First, each of the agents tested could induce chromosome breaks, resulting in the generation of acentric fragments that should lag subsequent to metaphase. The acentric fragments might then be encapsulated by the reforming nuclear membrane to generate micronuclei. Second, it is possible that the micronuclei are generated by a previously undescribed process occurring during S-phase when replication fork progression is inhibited.

The following experiments were performed to distinguish among these alternatives. First, we studied when micronuclei are generated during the cell cycle of Colo320 DM cells. The cells were synchronized at the G1/S boundary by a double thymidine block procedure, released into S-phase by thymidine removal, and progression into S-phase monitored using tritiated thymidine labeling. The number of micronuclei formed at each time after release was determined and plotted against the amount of tritiated thymidine incorporated into DNA. The data in Figure 3 clearly show that the number of micronuclei increase coincident with the progression of cells through S-phase. These micronuclei contain the C-myc DMs as indicated by their hybridization with a C-Myc FISH probe. Importantly, the number of micronuclei drops as the amount of DNA synthesis declines and the number of mitotic figures increases, perhaps because micronuclei are disassembled by the machinery involved in nuclear envelope breakdown. These data indicate that micronuclei can be generated during S-phase.

A second procedure involving confocal imaging, BrdU labeling and FISH was used to analyze whether cells engaged in DNA replication could generate micronuclei. We found that a substantial fraction of cells that appeared to be in interphase produced nuclear projections that labeled with propidium iodide, the C-Myc FISH probe, and BrdU (data not shown). This indicates that the projections were generated while DNA replication was occurring. The projections appear to be micronuclei precursors as many cells have a projection that is barely attached to the nucleus and appears ready to dislodge to become a micronucleus.

C. The p53 tumor suppressor affects micronucleation capacity

Loss of function of the p53 tumor suppressor is required for progression of cells into S-phase under the conditions that generate acentric chromosome fragments and that induce micronuclei (12, 13). We therefore determined whether micronucleation efficiency is related to p53 function. These experiments utilized normal diploid human fibroblasts (NDF), or isogenic derivatives that express the E6 gene product (from human papillomavirus type 16) which targets p53 for ubiquitin-mediated proteolysis (14).

Figure 4 shows that micronuclei are produced at low efficiency in NDF, or controls infected with a retrovirus that encodes the neomycin phosphotransferase gene. Incubation of these cells with HU (hydroxyurea, an inhibitor of ribonucleotide reductase), or PALA (n-phosphonacetyl-L-aspartate, a UMP synthesis inhibitor) does not increase micronucleation frequency. By contrast, the baseline level of micronucleation is increased approximately threefold in E6 expressing NDF. Micronucleation efficiency is increased five to tenfold above the baseline level of NDF, and approximately four-fold above that of E6-expressing NDF, when the optimum concentration of PALA is used for micronucleation. Increased micronucleation correlates with the capacity of PALA or HU to extend S-phase in these human fibroblasts. These data indicate that the presence of a functional p53 pathway limits micronucleation, or the production of the acentric fragments that induce micronuclei. We propose that loss of p53 function creates an environment which is permissive for the formation of acentric fragments, and also enables micronuclei to be generated at high efficiency. The mechanism by which p53 function minimizes micronucleation under growth limiting conditions remains to be elucidated. An important implication of these findings is that normal cells should not be subject to micronucleation under conditions that induce micronuclei in tumor cells lacking functional p53.

II. Micronucleation in tumors growing in vivo

A necessary step towards developing treatments targeted at tumors with DMs is to develop a rapid, simple method to identify DMs in tumor tissue without resorting to cytogenetics. This is required to identify the subset of DM containing tumors that would be candidates for such a protocol. We have begun to develop the required diagnostic procedure using xenografted primary tumors in nude mice as a model system. COLO 320DM cells (1×10^7) were injected into five week old nude mice (nu/nu). The tumor take rate was 90% (9/10) within 4 weeks, and tumors up to 40 mm diameter were generated. These tumors were dissected under sterile conditions and disaggregated by treatment with 0.15% collagenase 1a. Approximately 2×10^8 cells were obtained per tumor using this procedure.

The tumor cells were then analyzed for DMs and micronuclei. Disaggregated cells were fixed with methanol acetate and the frequency of micronucleus formation in vivo was examined by FISH using a C-Myc cosmid. Approximately 5% of nuclei exhibited micronuclei, suggesting that micronucleus formation occurs in vivo in the absence of extrinsic perturbations.

We next purified the micronuclei from dissected tumors. A sufficient number of micronuclei were produced from one tumor to enable purification using the same method we developed under this grant for cell lines (8). The ratio of C-Myc gene copy number was compared to that of a single copy gene to estimate the degree of purification. An approximately 1000 fold enrichment of C-Myc sequences was obtained, as observed for purification from a COLO 320DM cell line growing in vitro (8). A FISH probe made from the purified micronuclei using PCR was hybridized to a normal metaphase spread. The result was a clear and intense signal at chromosome 8q24, corresponding to the C-Myc locus. These data show that DMs can be detected and their chromosomal origin localized using the micronucleus purification and FISH procedure. The results also show that contamination of the tumor sample with normal cells does not present a problem that prevents a successful FISH analysis using the experimental model employed thus far. Of course, the generality of the procedure will need to be investigated using other tumor models. We also succeeded in purifying micronuclei from a short term culture of the dissected tumor. This short term culture strategy, or direct isolation of micronuclei from tumor specimens, may be able to be applied to a broad spectrum of cancer cell lines and tumor biopsy specimens.

III. Gene transfer mediated by micronuclei

The micronucleation flow diagram presented in Figure 2 raises the possibility that some micronuclei might be extruded through the cytoplasmic membrane. If this were to occur, it is conceivable that the triple membrane structure might be able to fuse to other tumor or normal cells, and deliver the contents of the micronucleus to the recipient cell. This might provide a means for increasing genetic instability and tumor cell heterogeneity.

We proposed to test the gene transfer ability of micronuclei as one of the aims of the modified statement of work. The first step to test this hypothesis involved building a vector whose transfer could be traced readily. We have established a model system using an artificial episomal viral vector in which replication is initiated at the replication origin of the Epstein-Barr virus (EBV) in combination with the EBNA-1 protein encoded by EBV. We showed that this vector replicates in human cells, such as COLO 320DM, and that it is incorporated into micronuclei along with the C-myc DMs present in COLO 320DM. The pEPBG vector also encodes the jellyfish protein GFP (green fluorescent protein) which is readily visualized after excitation of living cells with light of the appropriate wavelength. The pEPBG vector additionally encodes a gene engendering resistance to the cytotoxic drug blasticidin to enable selection of cells that have taken up the plasmid. To improve the utility of the vector, the GFP gene was fused in frame to a H2B gene so that chromosomes, including double minutes, could be visualized within living cells in real time.

We tested whether the components of the pEPBG vector function by electroporating it into COLO 320DM cells. Several clones with expressed transgenes were isolated by drug selection. All cells in each clone fluoresced brightly, indicating the stable maintenance of the transgene and expression of the GFP gene. FISH using two different colors for pEPBG and C-Myc demonstrated the co-localization of DMs and pEPBG episomes in 100% of micronuclei, as well as in metaphase spreads. This result is surprising and may be explained in two ways. First, recombination may have covalently joined the pEPBG vector with C-myc DMs. Alternatively, colocalization may involve a functional but non-covalent interaction between extrachromosomal molecules. Experiments to investigate these possibilities are in progress.

As expected, we found that histone-GFP localized to nuclei in the transfected cells. In mitotic cells, both mitotic chromosomes and DMs were visualized successfully by fluorescence microscopy. The staining pattern was indistinguishable from that obtained by DAPI, but a unique feature of this system is that it will enable us to observe the dynamics of chromosome movements and micronucleus formation in living cells. We plan to observe the process of micronucleus formation using a time course experiment, and to examine the effect of DNA synthesis inhibitors on micronucleus formation in real time. This experiment should enable us to gain insight into the dynamics of segregation of extrachromosomally amplified genes and expedite the development of rational and efficient chemotherapeutic strategies to eliminate extrachromosomally amplified genes.

IV. Analyzing the consequences of eliminating extrachromosomally amplified genes from tumor cell lines

This experiment was proposed contingent on availability of time, personnel and resources. We attempted to locate cell lines in which different classes of genes were amplified, such as growth factor receptors, cell cycle control proteins (e.g., cyclin D1, cdk4), or negative regulators of tumor suppressors (e.g., MDM2). We screened several cell lines and found that the amplified sequences had integrated into one or more chromosomal locations during extended passage in cell culture by other investigators. We are currently trying to establish some cell lines ourselves, and trying to obtain early clones of previously described cell lines.

Conclusions

The studies conducted in the last year provide several important advances that could facilitate the development of highly selective chemotherapeutic strategies targeted against molecular anomalies unique to cancer cells. The data summarized above provides evidence for a new mechanism of micronucleation involving nuclear budding during S-phase when replication fork progression is impeded. Interestingly, micronucleation was not stimulated in cells that have a functional p53 pathway using the same drugs that proved effective in tumor cells or isogenic p53-deficient normal cells. This raises the possibility that p53 acts, directly or indirectly, to determine whether acentric fragments will be generated under conditions that impede replication fork progression. As more than 50% of human tumors have mutated p53 genes, the micronucleation approach should be applicable to a majority of human neoplasms.

We also showed that many agents that interfere with DNA replication increase micronucleation. This greatly expands the spectrum of drugs that can be analyzed for their capacity to reduce the number of extrachromosomally amplified sequences.

The development of a vector system to label DNA fluorescently in living cells provides an opportunity to analyze micronucleation in real time. This should reveal more about the mechanism involved in nuclear budding during S-phase, and may avail us of methods to identify some of the gene products that participate. It may also provide a tool for screening for agents that induce micronuclei even more effectively than those analyzed thus far. The data also reveal that small viral replicons associate with DMs. Experiments in the next year will focus on determining whether this represents a covalent or non-covalent association, and

whether such association can be used to tag naturally existing DMs with marker episomes we construct in the laboratory. Tagging DMs with a selectable marker that can be scored readily on a cell by cell basis would enable us to assess the contribution of DM-encoded genes to cell growth, survival and tumorigenicity.

The studies performed thus far raise additional questions to be pursued in the next year. Our primary emphasis will be to elucidate more about the mechanism and consequences of micronucleation. We will use confocal microscopy to determine whether DMs are preferential targets for micronucleation because they occupy a specific site in the nucleus when they replicate. We will also attempt to determine whether chromosomes are excluded from micronuclei because they contain centromeres, telomeres, or both. We will pursue this goal by constructing linear replicating chromosomes driven by the EBV replication system to assess whether placing artificial telomeres on such molecules prevents their incorporation into micronuclei. If linear molecules with telomeres are incorporated into micronuclei, we would infer that centromeres most likely prevent molecules from being trapped in micronuclei. Micronucleation would then provide an assay for cloning functional centromeric sequences. We will also continue to analyze micronucleation *in vivo* using cell lines containing DMs encoding genes involved in different facets of cell cycle control. We will test some of the agents that proved most useful for micronucleation in DM elimination *in vitro* for such studies. This should enable us to determine the consequences of DM reduction on various parameters related to tumorigenicity and cell growth *in vitro* and *in vivo*.

Figure legends

1. Micronucleation is increased by DNA synthesis inhibitors under conditions that impede replication fork progression. Panel A lists the inhibitors used and the concentrations employed. Results are presented for COLO 320DM cells, although similar results have been obtained with PALA and HU in other cell lines, or primary cells deficient in p53 expression. COLO 320DM cells were treated for 3 days with aphidicolin (APH), coumarin (Cou), deferoxamin (Def.), DMSO, guanazole (Gua.), HU, nicotinamide (NA) or PALA at the concentration indicated in the figure. (A) The treated cells were fixed with methanol/acetic acid and hybridized with c-myc probe. The numbers of micronuclei that were stained heavily with c-myc probe were scored and expressed as frequency of micronuclei (%) relative to the number of interphase nuclei scored (more than 1000 for each point). (B) Cell cycle effects of drug treatments. The treated cells were recovered, fixed, immunostained for the incorporated BrdU and analyzed by flow cytometry as described in reference 11.
2. Models for formation of micronuclei. The two models presented are described in the text. The post-mitotic pathway is based on the extensive literature reporting reformation of nuclear envelopes around lagging chromatin associated with nuclear envelope reformation after metaphase. The model proposing nuclear budding is based on the data described in the progress report. Also shown is the possibility that micronuclei may be able to deliver their contents to recipient cells by a process involving fusion.
3. DM-containing micronuclei are formed through nuclear budding that occurs in S-phase. COLO 320DM cells were synchronized by incubation with excess thymidine (early S-phase arrest) followed by release in the absence of drugs. The culture was then divided into three portions, and released in the absence of any drug (A, D, G), the presence of nocodazole (B, E, H), or the presence of nocodazole and hydroxyurea (C, F, I). ³H-thymidine incorporation was determined from a portion of the culture (using trichloroacetic acid precipitation) (closed circles), and total nuclear budding (open circles) was quantified by counting DAPI stained slides (D, E, F). DM+micronuclei (closed circles) or DM+nuclear buds (open circles) were counted on slides hybridized with purified micronuclei probe (G, H, I). These numbers are

expressed as the frequency relative to the number of interphase nuclei scored (more than 1000 for each point).

4. Functional p53 pathway prevents the formation of micronuclei. Wild type (wt) WS1 human diploid fibroblast cells, WS1 cells infected with a control retrovirus encoding the neomycin phosphotransferase gene (neo), or the papilloma virus E6 gene (E6), were used. These cells were cultured in the absence or the presence of HU or PALA at the indicated concentration for 3 days. (A) Cell cycle effects of these drug treatments were examined by pulse labeling (30 min), with BrdU at the end of the drug treatments. The cells were recovered, fixed, immunostained for the incorporated BrdU and analyzed by flow cytometry. (B) Cells were grown on the cover slips, fixed by acetone and methanol, and stained with DAPI. The number of micronuclei were scored and expressed as the frequency of micronuclei (%) relative to the number of interphase nuclei scored (more than 1000 for each point).

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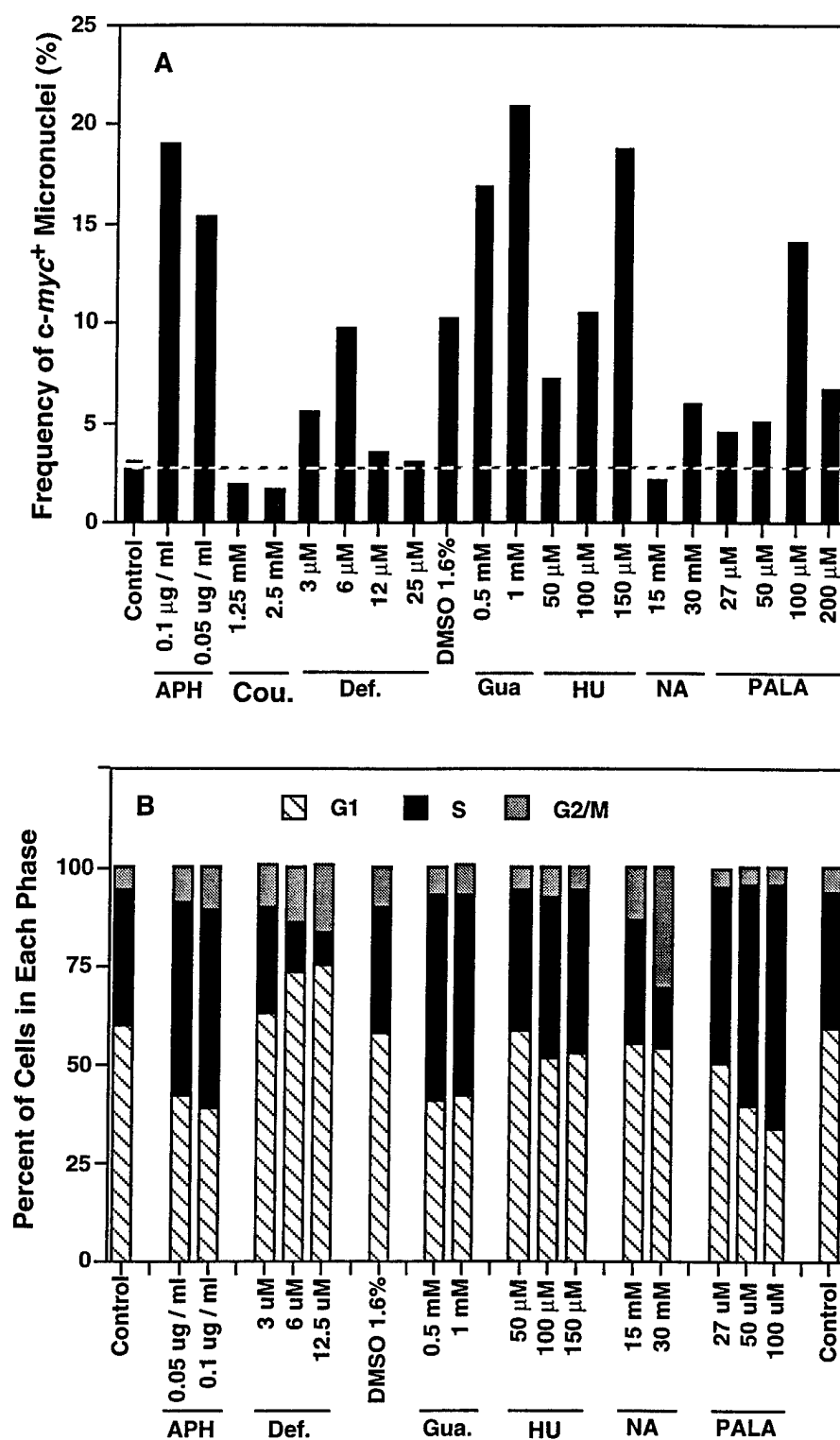


Figure 1

Figure 2

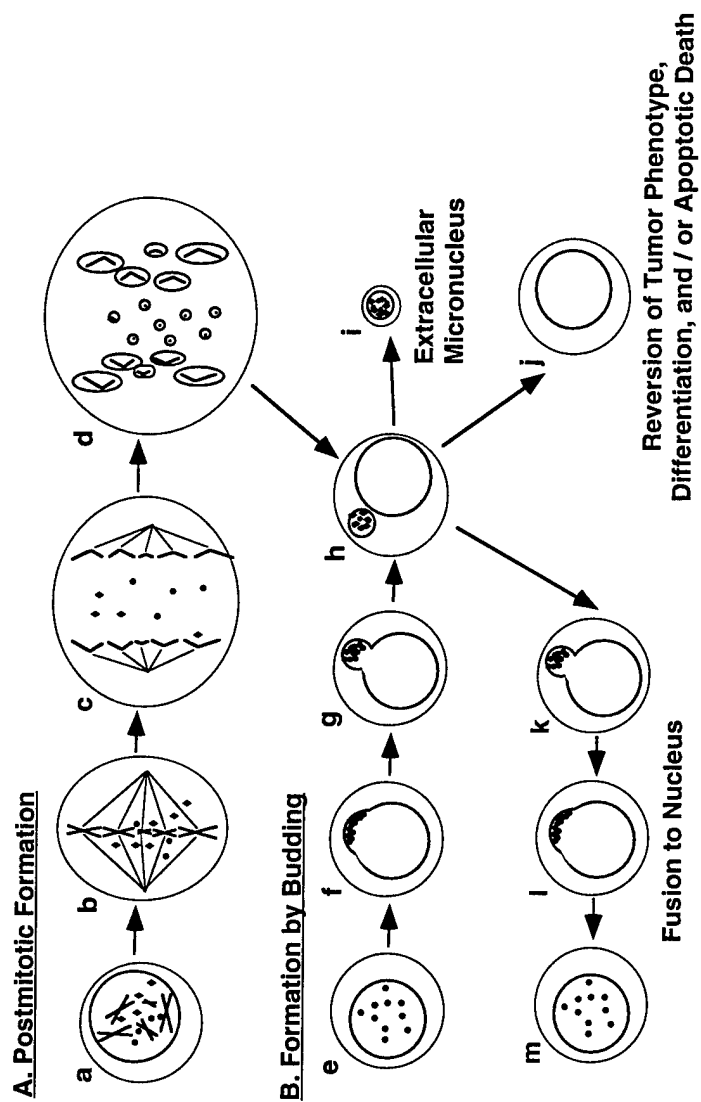


Figure 3

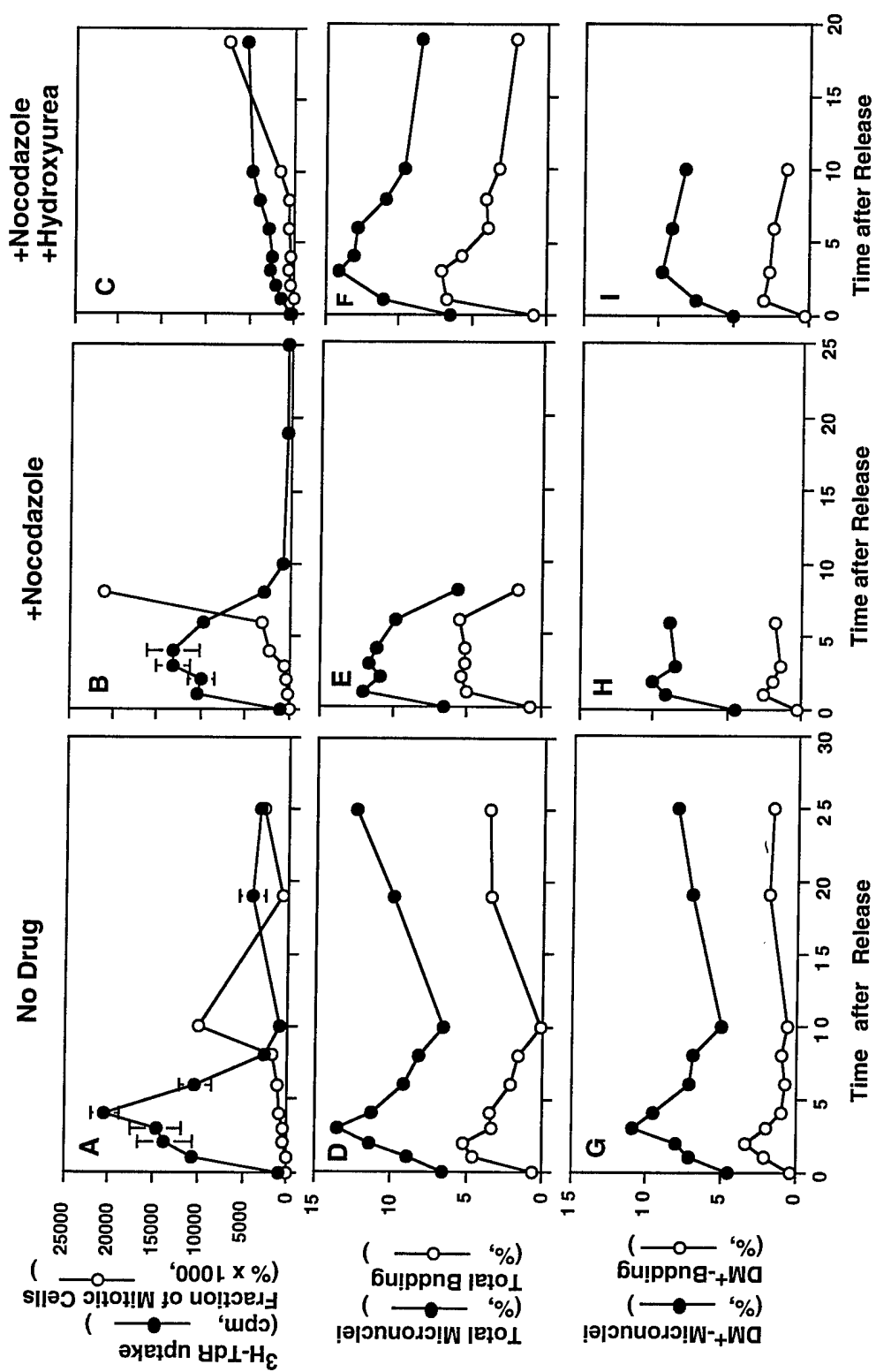
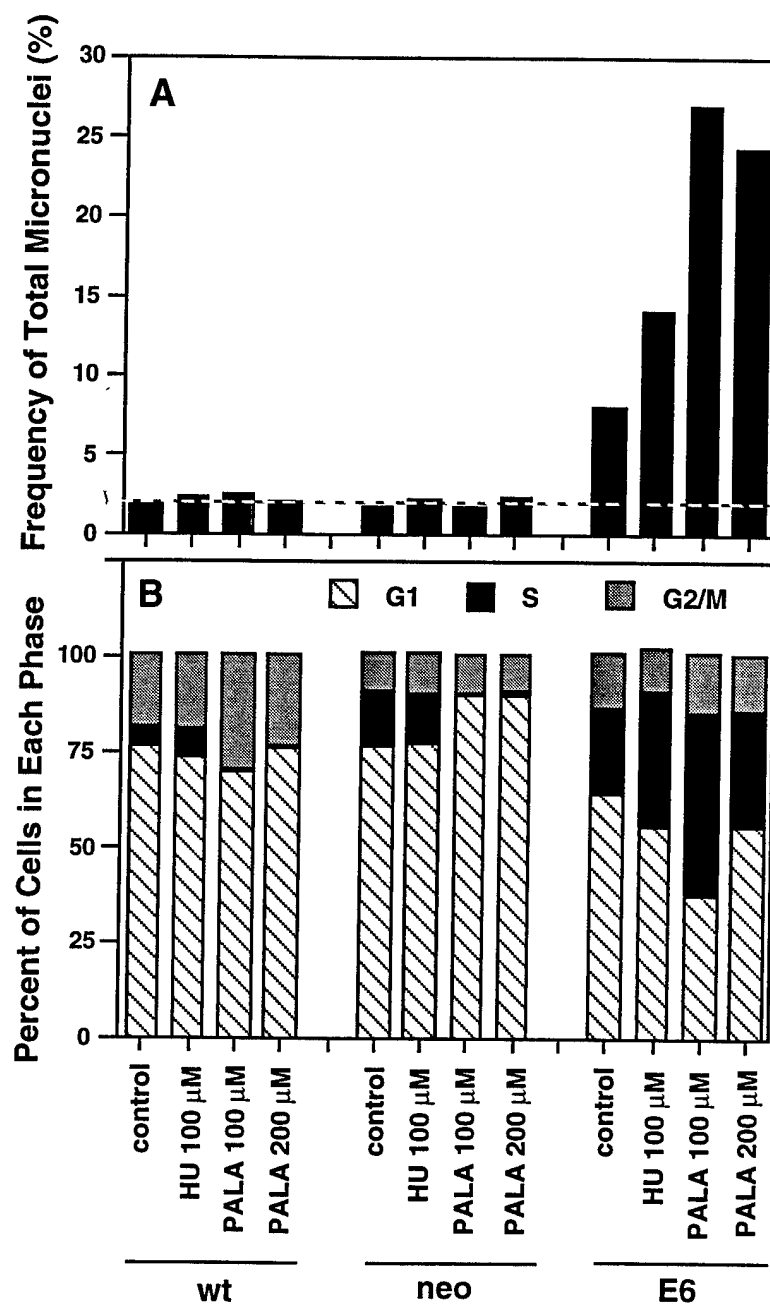


Figure 4



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